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Limiting replication initiation factors execute the temporal programme of origin firing in budding yeast

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Review timeline:

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Editorial Decision:	10 August 2011
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 August 2011

I wanted to give you an update on the review of your submission EMBOJ-2011-78890. We are currently still waiting for a delayed third report (a common occurrence at this time of the year). Since I will be away from the office for the next two weeks and you had indicated being in a competitive situation here, I have now carefully look through the two reports already at hand, and decided to contact you at this point with a preliminary decision. Both referees 1 and 2 are overall positive and would in principle support publication, pending adequate revision of a number of presentational and experimental concerns. Most of these points are pretty specific and well-taken, and we shall therefore be happy to consider a revised manuscript further for publication. I would thus like to invite you to start revising the manuscript according to the referees' comments and suggestions. I nevertheless have to stress that this is a preliminary decision and thus still subject to change should the last, missing report bring up serious additional concerns. Once the last report comes in, my colleagues will forward it to you and confirm this decision.

I should add that it is EMBO Journal policy to allow a single round of revision only, and that it is thus essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html).

Should you have any further questions regarding this decision or your revision, I will be happy to answer them upon my return to the office at the end of the month!

Sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, Zegerman and colleagues report the important observations that a subset of replication factors is limiting for origin activation in budding yeast and that overexpression of these factors (i) alters the replication timing program, (ii) activates S-phase checkpoints and (iii) impedes cell growth. It is now well established that replication origins are not activated simultaneously at the G1/S transition but rather fire sequentially throughout S phase. Many reasons have been invoked to explain why cells do not activate all origins upon entry into S phase, but the effect of premature origin activation could not be addressed so far because of the lack of an appropriate experimental system. Here, the authors convincingly show that overexpression of a set of limiting initiation factors is sufficient to induce the premature activation of late replication origins. When combined with the inactivation of the histone deacetylase Rpd3, another key regulator of replication timing, overexpression of these factors accelerates S phase progression and triggers a DNA replication stress response. Interestingly, they also show that checkpoint activation is suppressed when dNTP pools are increased by inactivation of Sml1, a key regulator of dNTP biosynthesis. They therefore conclude that dNTPs become limiting when too many origins are activated simultaneously, which would induce a replication stress. They also propose a model in which a set of limiting initiation factors is recycled after activation of early origins to promote the activation of late origins. This model is attractive and is consistent with the data presented in the manuscript. However, there are several important issues that need to be addressed prior to publication:
1) The title of the paper is rather cryptic. It is not clear for a non-specialized reader what competes for limiting initiation factors and how this competition would execute (determine?) a temporal program. Something like "Replication timing is determined by limiting initiation factors" would be more appropriate.
2) Overall, I found the results section of the manuscript very difficult to read. It would help to add subheadings, to reduce the number of supplementary figures and to move some part of the text to the discussion. It would also help to show a quantitation of the nascent DNA experiments, as in figure 3A (but the wt line is too light) with error bars when applicable.
3) Figure 1A: Since initiation factors like Dbf4 are only detected during a short window of time during S phase, it is important to show Sld2, Sld3 and Dpb11 levels during S phase, and not only in G1 or asynchronous cells.
4) Figure 1B: It is very surprising that initiation at ARS305 is hardly detectable in SSDD cells exposed to HU. The authors should either test other origins to show that this is a general phenomenon or remove this figure as these data in HU are not essential for the message of the paper.
5) Figure 3 is very complex and does not bring much to the paper. I would suggest moving the results on SSDDCS to supplementary materials.
6) Figure 6: Since the sml1 mutation increases dNTP levels rather modestly, the authors should overexpress RNR1 to test whether higher dNTP pools increase the viability of rpd3 SSDD cells.
7) Genome-wide studies from the Aparicio lab (Knott, 2009, Genes Dev. 23, 1077) have identified all the late origins that are repressed by Rpd3. The authors should refer to this study and check whether the late origins they have used are indeed controlled by Rpd3.
8) Page 10: the statement that "the average size of nascent DNA is smaller... strongly suggest that these strains deplete dNTPs at a faster rate" is too strong and should be toned down, unless if the authors directly show that dNTP levels are identical in both strains.
9) Page 11: "suggesting that the (e)ffect on the temporal program ..."

Referee #2 (Remarks to the Author):

The questions of how and why DNA replication timing is regulated are of increasing importance to those it the fields of DNA replication, genome stability, chromatin structure and epigenetic regulation. Many models propose that timing or origin firing is regulated by competition, with early origins competing more efficiently for one or more rate-limiting activators. Mantiero et al. use a candidate approach to identify such rate-limiting proteins. They find that 4 proteins - the initiation

proteins Sld2, Sld3 and Dpb11, and the Dbf4 regulatory subunit of the DDK kinase that regulates them - that are of relatively low abundance and that, when over expressed, advance the timing of origin firing. In series of well-conceived and well-executed experiments, the authors confirm their initial observation in several ways. They go on to show that perturbation of replication that they cause effects dNTP levels and compromises viability, suggesting a reason for regulating replication timing. All-in-all it is a technically solid paper that provides important insight into the mechanism by which replication timing is regulated in budding yeast. Furthermore, it fits well with other, albeit less extensive, results in other systems, suggesting a conserved mechanism (in general, if not in detail) for the regulation of eukaryotic replication timing. It will be of interest to a broad audience.

The manuscript could be improved by addressing the following points.

The method used to prepare the extracts probed in Figure 1a is not described. Given that some of these proteins are likely to be chromatin bound, it is important to know how the cells were lysed and if any insoluble material was removed by centrifugation.

The loading control used in Figure 1a is not very convincing. However, since only qualitative claims are made about protein levels from this figure, I do not think it is a serious problem.

On page 4, a sentence, or even just a phrase, about how the authors "confirmed the low abundance of Sld3, Dpb11 and Sld2 by measuring the number of molecules of each untagged protein per yeast cell" would help satisfy the curiosity that I suspect many readers will have.

I would be useful to have quantitation for Figure 3c, as well as 3a. And using uniform formatting for the annotation of the two panels would be an improvement.

It would be helpful to quantitate the replication time courses is Figure 4a and elsewhere, and show % replication vi time, instead of the raw histograms. It would allow some estimate for both the extent and the reproducibility of the effect to be made. Flow cytometry is notoriously prone to fluctuation, so reproducibility is a concern, especially given the modest extent of the effect. Representative flow-cytometry histograms (such as those show in 4a) could be moved to a supplemental figure.

The sentence "we found no increase in the speed of S phase upon over-expression of Dpb11 and Sld2 together with Sld3 and Dbf4 mutants that can not be inhibited by Rad53" is hard to parse. "we found no increase in the speed of S phase upon over-expression of Dpb11, Sld2, Sld3-A and Dbf4-A, the later two being serine-to-alanine mutants that can not be inhibited by Rad53."

It might be worth explicitly stating that the model presented in Figures 6c and S1 is applicable to any rate-limiting activator.

Additional correspondence 31 August 2011

We still have not received any comments from the outstanding third referee on your paper - despite multiple reminders sent to that referee by our editorial office in the meantime. Since the outstanding referee now had more than five weeks to raise any points or concerns, I do not see justification to wait any longer for their report. I am therefore happy to confirm my original decision based on referees 1 and 2, and would like to invite you to revise and resubmit your manuscript in light of only their comments now.

Looking forward to reading your revision

Best wishes,
Editor
The EMBO Journal

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1st Revision - authors' response 03 October 2011

Referee #1 (Remarks to the Author):

In this manuscript, Zegerman and colleagues report the important observations that a subset of replication factors is limiting for origin activation in budding yeast and that overexpression of these factors (i) alters the replication timing program, (ii) activates S-phase checkpoints and (iii) impedes cell growth. It is now well established that replication origins are not activated simultaneously at the G1/S transition but rather fire sequentially throughout S phase. Many reasons have been invoked to explain why cells do not activate all origins upon entry into S phase, but the effect of premature origin activation could not be addressed so far because of the lack of an appropriate experimental system. Here, the authors convincingly show that overexpression of a set of limiting initiation factors is sufficient to induce the premature activation of late replication origins. When combined with the inactivation of the histone deacetylase Rpd3, another key regulator of replication timing, overexpression of these factors accelerates S phase progression and triggers a DNA replication stress response. Interestingly, they also show that checkpoint activation is suppressed when dNTP pools are increased by inactivation of Sml1, a key regulator of dNTP biosynthesis. They therefore conclude that dNTPs become limiting when too many origins are activated simultaneously, which would induce a replication stress. They also propose a model in which a set of limiting initiation factors is recycled after activation of early origins to promote the activation of late origins. This model is attractive and is consistent with the data presented in the manuscript. However, there are several important issues that need to be addressed prior to publication:

We are very grateful to the referee for their comments and we address each of their points below.

1) *The title of the paper is rather cryptic. It is not clear for a non-specialized reader what competes for limiting initiation factors and how this competition would execute (determine?) a temporal program. Something like "Replication timing is determined by limiting initiation factors" would be more appropriate.*

We apologise if the title was not fully understandable. We have removed the word 'competition' to increase clarity.
Replication timing is a combination of the availability of limiting factors and the affinity of these factors for different origins. The word 'execute' in our title refers to the fact that while the timing of origin firing is determined in G1 phase in a process that is poorly understood, the temporal programme is actually executed in S-phase by a competition for limiting factors.

2) *Overall, I found the results section of the manuscript very difficult to read. It would help to add subheadings, to reduce the number of supplementary figures and to move some part of the text to the discussion. It would also help to show a quantitation of the nascent DNA experiments, as in figure 3A (but the wt line is too light) with error bars when applicable.*

We apologise if our results section was difficult to read. We have added subheadings to the results section. As suggested we have reduced the supplementary figures by removal of old supp figures 5, 8, 10 and 12 and reduction of old supp figures 6, 13, 14 and 16. We have also now extended the results and discussion sections within the format of this journal and we hope that this clarifies any ambiguities. In addition we have quantified all our 1D gels as suggested.

3) *Figure 1A: Since initiation factors like Dbf4 are only detected during a short window of time during S phase, it is important to show Sld2, Sld3 and Dpb11 levels during S phase, and not only in G1 or asynchronous cells.*

We show the levels of these proteins (untagged) during S-phase in Figure 4d (which is now Supp fig. 9d). The results are the same in a wild type strain.

4) Figure 1B: It is very surprising that initiation at ARS305 is hardly detectable in SSDD cells exposed to HU. The authors should either test other origins to show that this is a general phenomenon or remove this figure as these data in HU are not essential for the message of the paper.

We have tested other origins and this is a general phenomenon, likely due to the early activation of Rad53 in these experiments. We have removed the comments on this phenomenon from the paper and put in a longer exposure to avoid confusion.

5) Figure 3 is very complex and does not bring much to the paper. I would suggest moving the results on SSDDCS to supplementary materials.

We are keen to retain figure 3 as it to shows the effects of additional expression of Cdc45 and Sld7 and also shows that rpd3 affects dormant origin firing. We have reduced the number of panels in this figure to increase clarity and moved them to new supplementary figure 7, as suggested.
Figure 6: Since the sml1 mutation increases dNTP levels rather modestly, the authors should overexpress RNR1 to test whether higher dNTP pools increase the viability of rpd3 SSDD cells.
The sml1 mutation, which increases dNTP levels 2-3 fold, is sufficient to suppress the checkpoint activation that we see. As a result, this level of dNTP increase must be sufficient to suppress the majority of fork stalling events in our strains. We would therefore not expect even higher dNTP levels to have an impact on viability.

7) Genome-wide studies from the Aparicio lab (Knott, 2009, Genes Dev. 23, 1077) have identified all the late origins that are repressed by Rpd3. The authors should refer to this study and check whether the late origins they have used are indeed controlled by Rpd3.

We now reference this study on p.9. We do not expect our results to be directly comparable because the experiments in Knott 2009 were done in HU and compare rpd3 null strains to wild type cells. Our Rpd3 experiments are all in a normal S-phase and compare rpd3 null versus rpd3 null SSDD.

8) Page 10: the statement that "the average size of nascent DNA is smaller... strongly suggest that these strains deplete dNTPs at a faster rate" is too strong and should be toned down, unless if the authors directly show that dNTP levels are identical in both strains.

We apologise if we have over-stated our findings and we have changed that sentence accordingly (p.11).

9) Page 11: "suggesting that the (e)ffect on the temporal program ..."
We apologise for the typographical error.

Referee #2 (Remarks to the Author):

The questions of how and why DNA replication timing is regulated are of increasing importance to those it the fields of DNA replication, genome stability, chromatin structure and epigenetic regulation. Many models propose that timing or origin firing is regulated by competition, with early origins competing more efficiently for one or more rate-limiting activators. Mantiero et al. use a candidate approach to identify such rate-limiting proteins. They find that 4 proteins - the initiation proteins Sld2, Sld3 and Dpb11, and the Dbf4 regulatory subunit of the DDK kinase that regulates them - that are of relatively low abundance and that, when over expressed, advance the timing of origin firing. In series of well-conceived and well-executed experiments, the authors confirm their initial observation in several ways. They go on to show that perturbation of replication that they

cause effects dNTP levels and compromises viability, suggesting a reason for regulating replication timing. All-in-all it is a technically solid paper that provides important insight into the mechanism by which replication timing is regulated in budding yeast. Furthermore, it fits well with other, albeit less extensive, results in other systems, suggesting a conserved mechanism (in general, if not in detail) for the regulation of eukaryotic replication timing. It will be of interest to a broad audience.

We are very grateful to the referee for their comments and we address each of their points below.

The manuscript could be improved by addressing the following points.

The method used to prepare the extracts probed in Figure 1a is not described. Given that some of these proteins are likely to be chromatin bound, it is important to know how the cells were lysed and if any insoluble material was removed by centrifugation.

We apologise for this omission. We use a TCA precipitation and SDS/boiling solubilisation method to extract all cellular proteins. The protocol is now in the material and methods.

The loading control used in Figure 1a is not very convincing. However, since only qualitative claims are made about protein levels from this figure, I do not think it is a serious problem.

We apologise for the confusion. The loading control for the serial dilution of the same experiment in supplementary figure 2a is more definitive as it is an anti-PSTAIRE western. This data fully supports figure 1a.

On page 4, a sentence, or even just a phrase, about how the authors "confirmed the low abundance of Sld3, Dpb11 and Sld2 by measuring the number of molecules of each untagged protein per yeast cell" would help satisfy the curiosity that I suspect many readers will have.

We have now included an appropriate sentence at the bottom of p.4 to explain this in more detail.

It would be useful to have quantitation for Figure 3c, as well as 3a. And using uniform formatting for the annotation of the two panels would be an improvement.

We apologise for this over-sight and we now quantitate all 1D images for clarity.

It would be helpful to quantitate the replication time courses is Figure 4a and elsewhere, and show % replication vi time, instead of the raw histograms. It would allow some estimate for both the extent and the reproducibility of the effect to be made. Flow cytometry is notoriously prone to fluctuation, so reproducibility is a concern, especially given the modest extent of the effect. Representative flow-cytometry histograms (such as those show in 4a) could be moved to a supplemental figure.

Our flow cytometry experiments are always done sequentially on our FACS machine to reduce fluctuation and all experiments have been repeated 3 times or more to ensure the validity of our conclusions. Quantitiation of flow cytometry between experimental repeats is difficult due to the fluctuation mentioned in the referee comment, so we think it is more accurate to represent one experiment, rather than average several. To enhance the visual comparison of our flow results we now represent them as overlays and have moved the original histograms to supplementary data as suggested.

The sentence "we found no increase in the speed of S phase upon over-expression of Dpb11 and Sld2 together with Sld3 and Dbf4 mutants that can not be inhibited by Rad53" is hard to parse. "we found no increase in the speed of S phase upon over-expression of Dpb11, Sld2, Sld3-A and Dbf4-A, the later two being serine-to-alanine mutants that can not be inhibited by Rad53."

We apologise for the confusion in this statement and we have changed it accordingly (bottom of p.11).

It might be worth explicitly stating that the model presented in Figures 6c and S1 is applicable to any rate-limiting activator.

We now include a sentence in the figure legend of supplementary figure 1 to state this explicitly.

Pre-Acceptance letter 18 October 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that we can consider the study now in principle ready for publication in The EMBO Journal.

Before we will able to proceed with formal acceptance, there are a few presentational aspects which I would like to ask you to quickly address:
- please incorporate the minor modifications requested by referee 1 into the text
- when modifying the abstract, please try to make it as concise as possible. It should be maximally 175 words long not only in the submission system but also in the manuscript proper.
- we will need a brief Author Contribution and Conflict of Interest statement at the end of the manuscript text (preceding the reference section)

It should be sufficient if you simply send us a modified text document as email attachment in this case, and we would then replace it in our online submission system. I would appreciate if you could send us the re-revised text within the next few days to allow us to swiftly proceed with production and publication of the article.

Looking forward to receiving your final version,

Yours sincerely,
Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
The authors have properly addressed most of the issues raised by the Referees and have significantly improved the manuscript. In my opinion, the manuscript is now suitable for publication in EMBO Journal, once the following modifications have been made:
Abstract: The statement "... we demonstrate that the normal programme of origin firing prevents deoxyribonucleotide depletion..." is too strong. The authors show that artificial expansion of dNTP pools suppresses Rad53 activation in cells overexpressing initiation factors. This observation supports the view that the simultaneous activation of too many origins induces a depletion of dNTP pools, but this is not formally demonstrated in the manuscript. Since many other types of events leading to Rad53 activation can be suppressed by increased dNTP levels, the sentence in the abstract needs to be toned down.
Please cite references for "At this temperature the rate of replication is reduced and the delay between early and late origin firing is exacerbated..." (page 6) and "... at

least in part due to their hypo-acetylated chromatin state" (page 9).
Page 11: "...the average size of nascent DNA is smaller in strains that allow the early firing...". Please define these strains.
Page 12: "we measured late/dormant origin firing in a strain lacking SML1, where the activation of the checkpoint is suppressed". This sentence is misleading. The authors refer to a strain lacking SML1 and overexpressing initiation factors. Moreover, the spontaneous activation of Rad53 is suppressed under these conditions, not checkpoint activation in general.

2nd Revision - authors' response 17 October 2011

I enclose a final version of the manuscript with the changes you have outlined. I have summarised them below for your convenience.

Please incorporate the minor modifications requested by referee 1 into the text
See list of changes below

- when modifying the abstract, please try to make it as concise as possible.
It should be maximally 175 words long not only in the submission system but also in the manuscript proper.

It is now 175 words long.

- we will need a brief Author Contribution and Conflict of Interest statement at the end of the manuscript text (preceding the reference section)

This is now included

Referee #1

(Remarks to the Author)
The authors have properly addressed most of the issues raised by the Referees and have significantly improved the manuscript. In my opinion, the manuscript is now suitable for publication in EMBO Journal, once the following modifications have been made:
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We have replaced the phrase " prevents deoxyribonucleotide depletion", with " prevents checkpoint activation " which is what we show.

Please cite references for "At this temperature the rate of replication is reduced and the delay between early and late origin firing is exacerbated..." (page 6)

This is our (unpublished obervations) and we have included this phrase

"... at least in part due to their hypo-acetylated chromatin state" (page 9).

We have included a reference from the Grunstein lab (Suka et al 2001) which
shows HML is hypoacetylated.

Page 11: "...the average size of nascent DNA is smaller in strains that allow the early firing...". Please define these strains.

We have altered this sentence to make sure the strains we used are clear.

Page 12: "we measured late/dormant origin firing in a strain lacking SML1, where the activation of the checkpoint is suppressed". This sentence is misleading. The authors refer to a strain lacking SML1 and overexpressing initiation factors.
Moreover, the spontaneous activation of Rad53 is suppressed under these conditions, not checkpoint activation in general.

We have clarified this sentence accordingly to "firing in the SSDD strain lacking SML1, where the activation of Rad53 is suppressed"